J. Physiol. (1977), **267**, *pp.* 497–518
With 9 text-figures
Printed in Great Britain

THE EFFECTS OF STRONTIUM AND BARIUM IONS AT SYNAPSES IN SYMPATHETIC GANGLIA

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(*Received 12 October 1976*)

SUMMARY

1. A study has been made of the effects of Sr^{2+} and Ba^{2+} ions at synapses in isolated superior cervical ganglia of guinea-pigs. Intracellular recordings of membrane potential were made from ganglion cells in the presence of different concentrations of Ca^{2+}, Sr^{2+} and Ba^{2+} ions.

2. The addition of Sr^{2+} (2–5 mM) caused little change in resting membrane potential; in contrast, Ba^{2+} (1–6 mM) always depolarized the cells and prolonged the duration of action potentials.

3. The resting frequency of spontaneous miniature excitatory postsynaptic potentials (min. e.p.s.p.s) was briefly accelerated by the addition of either Sr^{2+} or Ba^{2+}; but subsequently returned to about control levels.

4. Following replacement of Ca^{2+} by Sr^{2+}, e.p.s.p.s could always be evoked during repetitive stimulation of preganglionic axons at a fixed latency after the nerve impulses ('phasic' transmitter release). Replacement of Ca^{2+} by Ba^{2+} produced many asynchronous e.p.s.p.s during trains of impulses ('residual' transmitter release).

5. By analysis of the interaction between Sr^{2+} and Ca^{2+}, Sr^{2+} was shown to have a partial agonist action on 'phasic' transmitter release. The same analysis applied to Ba^{2+} failed to demonstrate either a partial agonist or an antagonist action.

6. Both Sr^{2+} and Ba^{2+} prolonged e.p.s.p.s. Changes in Sr^{2+} could mainly be attributed to its effect on cell input resistance; Ba^{2+} may also prolong the time course of transmitter release.

7. The increased frequency of min. e.p.s.p.s which occurs during repetitive stimulation was potentiated by both Sr^{2+} and Ba^{2+}, Ba^{2+} being about twice as potent as Sr^{2+}. This activation of 'residual' transmitter release is independent of the action of these ions on 'phasic' release.

8. It is concluded that the reported maintenance by Ba^{2+} of acetylcholine output from perfused ganglia results from the asynchronous release of large numbers of quanta during trains of impulses.

INTRODUCTION

The depolarization-induced influx of Ca^{2+} ions into presynaptic nerve terminals results in the almost synchronous release of a number of quanta of transmitter (Katz, 1969). This process is antagonized by many divalent cations, e.g. Mg^{2+} (Jenkinson, 1957), Mn^{2+} (Balnave & Gage, 1973), Co^{2+} and Ni^{2+} (Kita & van der Kloot, 1973), in a manner consistent with competition for the same receptor sites. The only ion which has consistently been shown to replace Ca^{2+} in the activation of this 'phasic' (Miledi & Thies, 1971) form of transmitter release is Sr^{2+} (Miledi, 1966; Dodge, Miledi & Rahamimoff, 1969). Analysis of the action of Sr^{2+} ions at the neuromuscular junction (Meiri & Rahamimoff, 1971) has shown Sr^{2+} to be less effective than Ca^{2+}, having the characteristics of a partial agonist of the 'Ca receptor'. The action of Ba^{2+} ions on this process is unclear (see Hubbard, 1973). Focal depolarization of the neuromuscular junction preceded by iontophoretic application of Ba^{2+} was occasionally followed by the release of quanta of acetylcholine (ACh) (Miledi, 1966), while at the squid giant synapse, only a temporary restoration of synaptic potentials followed the addition of Ba^{2+} ions to a Ca-free medium (Katz & Miledi, 1969). On the other hand, Ba^{2+} ions have been reported to act as an effective substitute for Ca^{2+} ions to maintain levels of ACh output from perfused superior cervical ganglia during repetitive stimulation of the preganglionic nerve (Douglas, Lywood & Straub, 1961).

In an attempt to resolve these anomalies, the effects of Ba^{2+} ions on transmitter release have been examined at synapses in isolated sympathetic ganglia, and compared with the effects of Sr^{2+} ions. Excitatory postsynaptic potentials (e.p.s.p.s), recorded in ganglion cells with intracellular micro-electrodes, have been studied in the presence of different concentrations of Ca^{2+} ions, and the effects of addition of Sr^{2+} and Ba^{2+} ions on these responses have been analysed. The results suggest that Ba^{2+} ions do not replace Ca^{2+} to support the 'phasic' release of quanta of ACh which follows each nerve impulse in the preganglionic axons. A separate action of both Sr^{2+} and Ba^{2+} ions is to potentiate the asynchronous release of quanta of ACh during trains of stimuli. This seems most likely to be the source of the ACh which appears in the venous effluent of perfused sympathetic ganglia during preganglionic stimulation.

A preliminary report of these results has already been published (McLachlan, 1976).

METHODS

Isolated superior cervical ganglia from guinea-pigs (150–200 g) were used in all experiments. Ganglia were dissected from animals anaesthetized with urethane (1·0–1·5 g/kg I.P.), and pinned out in a Perspex organ bath of 5 ml. capacity. The

preparation was bathed initially in a modified Krebs solution of the following composition (mM): Na 151, K 4·7, Mg^2 1·2, Cl 144·4, H_2PO_4 1·3, HCO_3 16·3, glucose 7·8, gassed with 95% O_2, 5% CO_2. After 15–30 min in this nominally Ca^{2+}-free solution (probably < 0·02 mM, Miledi & Thies, 1971), different concentrations of $CaCl_2$ (0·5–4 mM), $SrCl_2$ (2–5 mM) and $BaCl_2$ (1–6 mM) were added to the perfusate as indicated in the Results, no adjustments being made for the small changes in tonicity. Following the initial period of wash-out of Ca^{2+} from the ganglia, subsequent solution changes resulted in steady-state release conditions after 5–10 min (see also Dodge *et al.* 1969; Meiri & Rahamimoff, 1971). The bathing solution was maintained at 34–35° C and flowed continuously through the bath at 3–6 ml./min.

The cervical sympathetic trunk was split longitudinally and about $\frac{1}{3}$ of the nerve was stimulated, by means of a suction electrode, with pulses of 0·01–0·3 msec duration and 2–15 V amplitude. Membrane potentials were recorded from ganglion cells using glass micro-electrodes filled with 2 M-KCl, and having resistances of 40–80 MΩ. The signals were led via a high impedance unity gain preamplifier to an oscilloscope and photographed on moving film. In most experiments, cells were impaled after the addition of 0·5, 1 or 2 mM-$CaCl_2$ to the perfusate. In these solutions, recordings were made only if a resting membrane potential $\geqslant$ 55 mV was established. Lower resting potentials were tolerated in Ca^{2+}-free solutions. The stimulus was then adjusted until only one preganglionic axon was being excited. In most cases, a synapse thus identified was rejected for analysis if the amplitude of the facilitated e.p.s.p. during trains of stimuli did not remain sub-threshold for an action potential. Usually, mean e.p.s.p. amplitudes were much smaller than 10 mV, so that the error in the determination of the mean quantal content (m) from the ratio of mean e.p.s.p. amplitude to mean miniature e.p.s.p. amplitude was likely to be small.

In order to estimate m in steady-state conditions of release at ganglionic synapses, the following stimulating procedures were used so as to standardize the degree of facilitation of the presynaptic axons (see McLachlan, 1975*a*). Single supramaximal stimuli were delivered to the preganglionic axons every 15 sec from the start of the experiment. Once an impalement was achieved and the stimulus adjusted (as described above), this frequency of stimulation was maintained. At 15 min intervals, cell membrane properties were determined and then the axon was stimulated with two trains of 100 stimuli, the first at 2 Hz, the second at 10 Hz. In preliminary experiments in 2 mM-Ca^{2+}, reproducible estimates of m (during a steady state of release at each frequency) could be made at 15 min intervals over several hours. Solution changes were made immediately after a recording period; under these conditions, the initial levels of transmitter release were readily restored on return to the original bathing solution.

RESULTS

Effects on membrane properties

Resting membrane potentials were unaltered by changes in Ca^{2+} concentration between 0·5 and 4 mM, by the addition of Sr^{2+} (2–5 mM) to solutions containing added Ca^{2+} (0·5–2 mM) or by the replacement of 2 mM-Ca^{2+} by 2 mM-Sr^{2+}. In contrast, ganglion cells always depolarized by more than 10 mV over 5–10 min following the addition of Ba^{2+} (1–6 mM); in most cases, short bursts of action potentials were initiated from an unstable depolarized level of membrane potential (Fig. 1*a*). Under these conditions, it was usually difficult to maintain the impalements. The cells were therefore hyperpolarized to their original resting

membrane potentials by currents passed via the recording micro-electrodes. If the hyperpolarizing currents were reduced slowly after more than 10 min in Ba-containing solutions, spontaneous action potentials were rarely initiated at the resting membrane potential although slow oscillations were present (Fig. 1*b*). The degree of depolarization was dependent on the Ba^{2+} concentration, being less rapid and less severe if Ca^{2+} was present; this membrane stabilizing effect of Ca^{2+} was greater

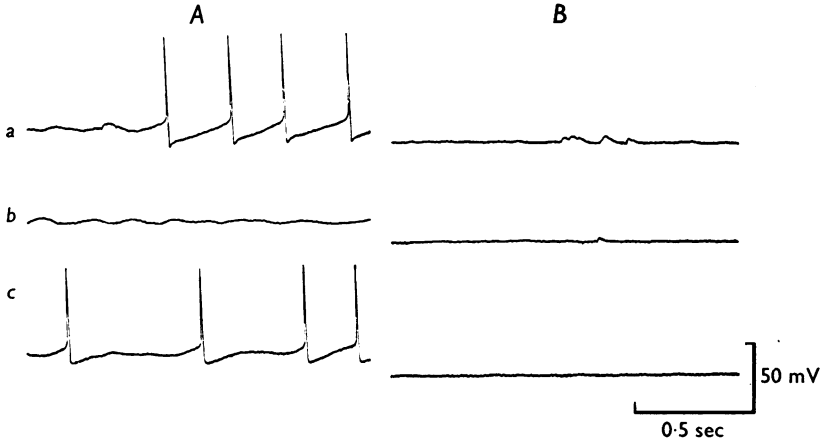

Fig. 1. The effect of Ba^{2+} ions on membrane potential. Changes in membrane potential recorded in a cell of the guinea-pig superior cervical ganglion. *A*, at cell resting potential; *B*, after hyperpolarization of the cell to the original resting potential (58 mV) in control solution (containing 0·5 mM-Ca^{2+}). *a*, 3 min after the addition of 5 mM-Ba^{2+}; *b*, 15 min after *a*; *c*, 4 min after adding 5 mM-Ba^{2+} to Ca^{2+}-free perfusate. Initially Ba^{2+} depolarized the membrane and bursts of action potentials were initiated; at this time bursts of e.p.s.p.s were observed if Ca^{2+} was present. After 15 min the resting membrane potential was unstable, but action potential firing had ceased; single min. e.p.s.p.s were observed at low frequency.

at 2 mM than at 0·5 mM (see also Laskowski & Thies, 1972). Cells were also depolarized following the removal of added Ca^{2+}, but to a lesser extent and spontaneous action potential firing was less common. All measurements of membrane properties and transmitter release were therefore made with membrane potentials held at the resting potentials measured in solutions containing at least 0·5 mM-Ca^{2+}.

Increasing the concentration of either Ca^{2+} (from 0 up to 4 mM) or Sr^{2+} (from 2 to 5 mM) always increased the cell input resistance, R_{in}, the magnitude of the increase increasing with the total concentration of these ions. In Ca^{2+}-free solutions containing 2 mM-Ba^{2+}, R_{in} was always lower than that determined in the same cell in 2 mM-Ca^{2+} (Ba^{2+}-free) solution

(n = 5). When 2 mM-Ba^{2+} was added in the presence of either 0·5 or 2 mM-Ca^{2+}, R_{in} increased in three of nine cells, did not change in five others and fell slightly (by 13 %) in the remaining cell. Significant changes in cell capacitance, C, could not be detected.

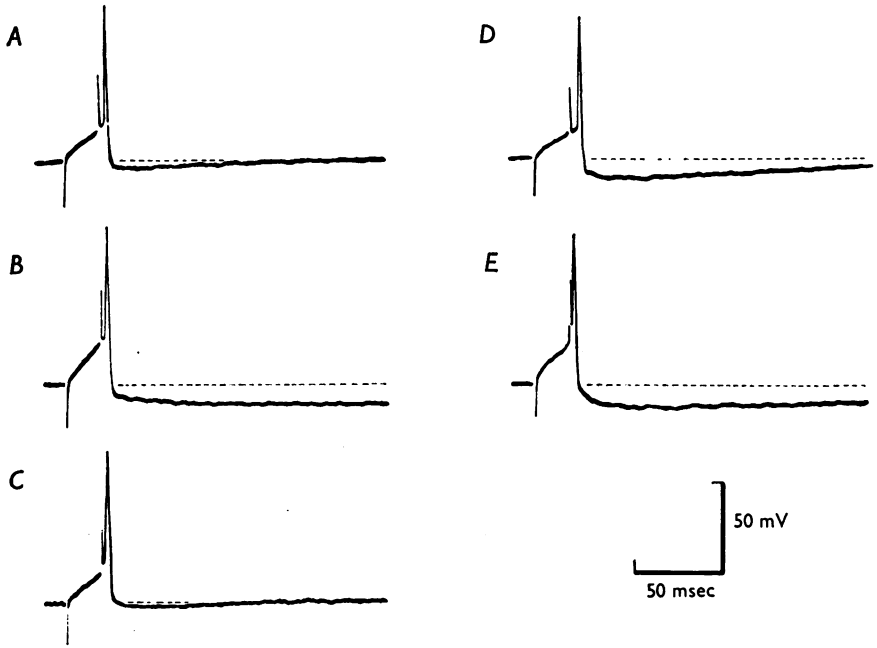

Fig. 2. The effect of the addition of Sr^{2+} on action potentials initiated by brief (20 msec) depolarizing pulses. *A*, *B*, *C*, recorded in one cell; *A*, in 0·5 mM-Ca^{2+} (0·33 nA current intensity), *B*, in 0·5 mM-Ca^{2+} + 5 mM-Sr^{2+} (0·4 nA), *C*, in 0-Ca^{2+}, 0-Sr^{2+} (0·3 nA). *D*, *E*, recorded in another cell; *D*, in 2 mM-Ca^{2+} (0·34 nA), *E*, in 2 mM-Ca^{2+} + 5 mM-Sr^{2+} (0·51 nA). Dashed lines indicate resting membrane potentials. At the higher Ca^{2+} concentration and following the addition of Sr^{2+}, afterhyperpolarizations were larger and more prolonged.

Action potentials. The amplitude of action potentials was not affected if Ca^{2+} was raised from 0·5 to as high as 4 mM, or if Sr^{2+} (up to 5 mM) was added to solutions containing Ca^{2+} (0·5 or 2 mM). However, such solution changes raised the threshold for action potential initiation and augmented and prolonged the hyperpolarizations following them (Fig. 2). In Ca^{2+}-free solutions or those containing Ba^{2+} (1–6 mM), the peak of the action potentials was slightly lower (< 10 %), their firing threshold was reduced and afterhyperpolarizations were virtually abolished (Figs. 2*C*, 3*B*, *C*). In addition, the half-width of the action potential was increased

from about 2 msec to a maximum of 2·8 msec by the removal of Ca^{2+} and to a maximum of 4 msec by the addition of Ba^{2+} (1–6 mM) (see Fig. 3).

It appears that Sr^{2+} ions and Ca^{2+} ions have similar actions on membrane properties consistent with membrane stabilization (Shanes, 1958). Ba^{2+} ions affect the membrane in a way similar to that which follows the removal of Ca^{2+} ions; most of these actions are consistent with a decrease

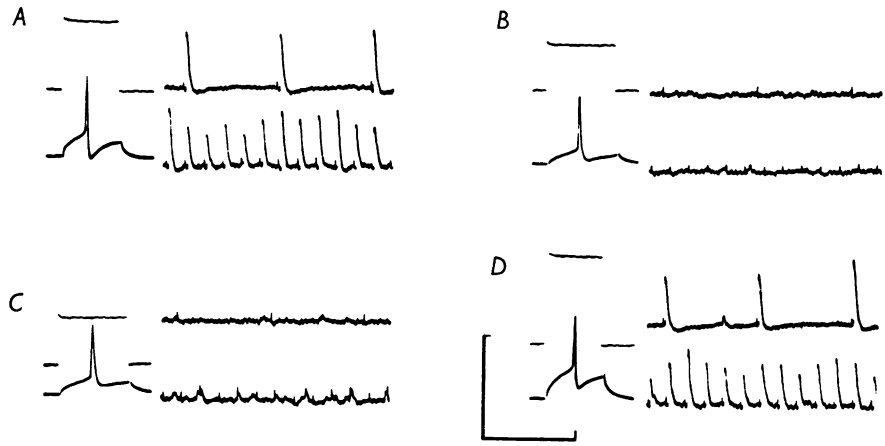

Fig. 3. The effect of Ba^{2+} on action potentials and the release of ACh. Action potentials recorded at low gain (d.c.) together with monitored current (on the left of each panel), and e.p.s.p.s recorded at high gain (a.c.) during steady-state release at 2 and 10 Hz (on the right of each panel). Bathing solutions contained: *A*, 2 mM-Ca^{2+}, *B*, 0-Ca^{2+}, 0-Ba^{2+}, *C*, 2 mM-Ba^{2+}, 0-Ca^{2+}, and *D*, return to 2 mM-Ca^{2+}, 0-Ba^{2+}, recorded at 15 min intervals in the same cell. Vertical calibration represents 0·6 nA current, 100 mV low-gain for action potentials, and 5 mV high-gain for e.p.s.p.s; horizontal calibration represents 45 msec for low-gain traces, and 500 msec for high-gain traces. E.p.s.p.s evoked by each nerve impulse disappeared in Ca^{2+}-free solution, although some min. e.p.s.p.s occurred asynchronously with the stimuli. In the presence of Ba^{2+}, many more min. e.p.s.p.s were evoked during repetitive stimulation, but e.p.s.p.s at the appropriate latency were not seen.

in permeability to K^+ ions (Werman & Grundfest, 1961), although the effects on R_{in} suggest that this cannot be the only effect of Ca^{2+} removal. The effects of reduced Ca^{2+} concentration differ in some ways from those reported for other neurones (see, for example Frankenhaeuser & Hodgkin, 1957; Frankenhaeuser, 1957; Barrett & Barrett, 1976), although they resemble those seen in cardiac muscle (Hoffman & Suckling, 1956); these effects require further investigation.

Effects on spontaneous release of ACh

The resting frequency of spontaneous miniature e.p.s.p.s (min. e.p.s.p.s) in Ca^{2+}-free solutions was very low, in some cases none being detected during 15 min of continuous observation. Min. e.p.s.p.s were observed more frequently in 2 mM-Ca^{2+} than in 0·5 mM-Ca^{2+}. Although resting min. e.p.s.p. frequencies in sympathetic ganglia are low (< 0·1 Hz, Blackman & Purves, 1969; McLachlan, 1974), they apparently increase with increases in Ca^{2+} concentration as reported at the neuromuscular junction (see Hubbard, 1973).

Equimolar replacement of 2 mM-Ca^{2+} by Sr^{2+} or Ba^{2+} produced no detectable changes in resting min. e.p.s.p. frequency. However, further increases in Ba^{2+} concentration to 4 or 6 mM (in the absence of added Ca^{2+}) increased spontaneous release rates (see also Elmqvist & Feldman, 1965), to an extent greater than that seen if Ca^{2+} was raised to 4 mM.

When either Sr^{2+} or Ba^{2+} (2–5 mM) was added to solutions containing 0·5 or 2 mM-Ca^{2+}, a transient small increase (two to threefold) in min. e.p.s.p. frequency occurred over about 5 min. Subsequently, resting frequency returned to near control levels. Although this initial increment may have been due to the small increase in osmolarity, it seems unlikely that this can account for subsequent effects of these ions as changes in min. e.p.s.p. frequency were not maintained (Furshpan, 1956).

On some occasions when 2 or 5 mM-Ba^{2+} was added in the presence of 0·5 or 2 mM-Ca^{2+}, large spontaneous e.p.s.p.s occurred in bursts (Fig. 1*a*). After about 10 min exposure to Ba^{2+}, these were no longer detected (Fig. 1*b*). As bursts of e.p.s.p.s were never observed in the absence of Ca^{2+} (Fig. 1*c*), it seems likely that these resulted from spontaneous firing of nerve impulses in the presynaptic terminals.

Effects on quantal content of e.p.s.p.s evoked by preganglionic stimulation

Strontium. Stimulation of preganglionic axons always evoked e.p.s.p.s in ganglion cells perfused with a Ca^{2+}-free solution containing 2 mM-Sr^{2+} (twenty-three cells in four ganglia). However, it was often necessary to stimulate a single axon at high frequency (10 Hz) before e.p.s.p.s could be detected.

At two synapses, the binomial statistic parameters, m, p and n (Johnson & Wernig, 1971; Robinson, 1976), were determined initially in 2 mM-Ca^{2+}, and compared with estimates made at the same synapses in 2 mM-Sr^{2+}. In both cases, the quantal content, m, was reduced in Sr^{2+}, and the values of both p and n were lower than the respective values determined in normal Ca^{2+} (see Table 1). At two other synapses examined only in 2 mM-Sr^{2+}, the estimate of p at 10 Hz was < 0·2; this value is low compared

with that normally determined at this frequency at similar synapses in normal Ca^{2+} concentrations (McLachlan, 1975*a*). Because of the low values of p estimated in Sr^{2+} and the small size of the samples, standard errors of these estimates are likely to be large and quantitative interpretation of these data is very unreliable (see McLachlan, 1975*b*).

It seems reasonable to conclude that Sr^{2+} can support the 'phasic' release of ACh, but its effectiveness is much less than that of Ca^{2+}, as reported for the neuromuscular junction (Dodge *et al.* 1969; Meiri & Rahamimoff, 1971).

Barium. In ganglia perfused with Ca^{2+}-free solutions containing 2 mM-Ba^{2+}, the only detectable release of transmitter was in the form of a few min. e.p.s.p.s occurring during supramaximal high-frequency stimulation of the preganglionic axons (fifteen cells in four ganglia). In no instance was it possible to identify an e.p.s.p. at a normal latency after the nerve impulse. In two of these ganglia, e.p.s.p.s were evoked at normal latencies within a minute of adding 2 mM-Ca^{2+} to the bathing solution, although none were observed in the same cells in Ba^{2+} solution before exposure to Ca^{2+} ions. In the two remaining ganglia, both of which had been in Ba^{2+} solution for more than 4 hr, there was no recovery of 'phasic' release after the introduction of Ca^{2+}, although resting and action potentials recovered to normal in a few minutes and increased numbers of min. e.p.s.p.s were observed during high-frequency stimulation. These results suggested that Ba^{2+} was ineffective in the support of 'phasic' transmitter release, although it was possible that prolonged exposure to Ba^{2+} had interfered with normal presynaptic function.

Following equimolar substitution of 2 mM-Ca^{2+} with Ba^{2+}, stimulation at low frequency ($< 0{\cdot}1$ Hz) failed to evoke responses but a few e.p.s.p.s occurred at an appropriate latency after the stimulus during trains (five cells) (Fig. 4). In contrast, even high-frequency stimulation failed to evoke e.p.s.p.s at the appropriate latency if a period of exposure to Ca^{2+}-free solution preceded the addition of 2 mM-Ba^{2+} (seven cells) (see Fig. 3). Whether or not Ca^{2+} had been washed out of the preparation, large numbers of min. e.p.s.p.s appeared asynchronous with the stimuli (Figs. 3*C*, 4*B*, see also Fig. 6*B*); the number of these 'residual' (Miledi & Thies, 1971) min. e.p.s.p.s in 2 mM-Ba^{2+} was considerably greater than that observed in Ca^{2+}-free, Ba^{2+}-free solutions (Fig. 3*B*). Under these conditions, measurements of peak changes in membrane potential in successive intervals following each nerve impulse at 10 Hz were used as estimates of transmitter output and showed no variation in release with time.

Two possibilities arise: (*a*) Ba^{2+} activates 'phasic' release almost as effectively as Ca^{2+}, but release occurs over a very prolonged time course (Alnaes, Meiri, Rahamimoff & Rahamimoff, 1974), or (*b*) Ba^{2+} is not an

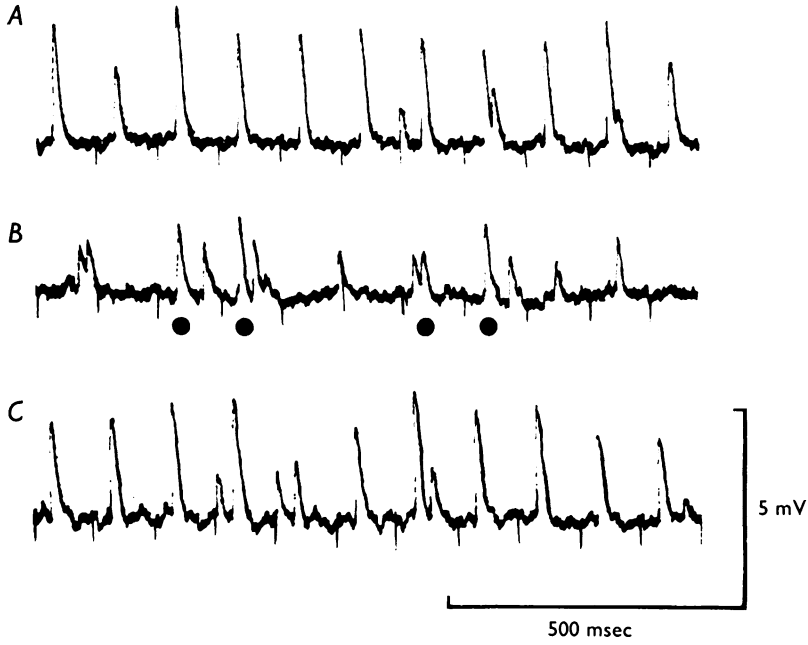

Fig. 4. The effect of equimolar substitution of Ca^{2+} by Ba^{2+}. E.p.s.p.s recorded during steady-state release at 10 Hz at a single synapse. *A*, 2 mM-Ca^{2+}; *B*, 30 min after changing to 2 mM-Ba^{2+}; *C*, return to 2 mM-Ca^{2+} after 60 min in Ba^{2+}. Only a few e.p.s.p.s (indicated by the dots) appear at an appropriate latency after the nerve impulses in Ba^{2+} solution. The total number of quanta released in these sample traces is estimated at 39, 23, and 41 respectively.

TABLE 1. Estimates of statistical release parameters during steady-state release at 10 Hz in Sr^{2+} solutions

Axon	Solution containing	Mean e.p.s.p. amplitude (mV)	S^2	Mean min. e.p.s.p. amplitude (mV)	σ^2	m	p	n	m_0
I	2 mM-Ca^{2+}	3·06	0·82	0·41	0·018	7·33	0·43	15·08	—
	2 mM-Sr^{2+}	0·16	0·07	0·44	0·022	0·37	0·13	2·90	0·40
II	2 mM-Ca^{2+}	5·49	1·73	0·65	0·051	8·41	0·64	13·17	—
	2 mM-Sr^{2+}	0·91	0·56	0·69	0·053	1·32	0·23	5·77	1·42
III	2 mM-Sr^{2+}	1·62	0·93	0·57	0·032	2·86	0·09	32·46	2·83
IV	2 mM-Sr^{2+}	0·85	0·38	0·48	0·029	1·77	0·19	9·25	1·68

S^2, variance of e.p.s.p.s; σ^2, variance of min. e.p.s.p.s; m, p, n, binomial release parameters (determined by the method of Robinson, 1976); m_0, estimate of mean quantal content based on Poisson statistics = ln (number of impulses/number of failures), providing comparable estimates of m when failures occur and $P < 0·2$.

agonist of 'phasic' release but powerfully activates the 'residual' release of transmitter evoked during trains of impulses.

Interaction between agonists

Strontium. Meiri & Rahamimoff (1971) analysed the action of Sr^{2+} ions on transmitter release at the neuromuscular junction by studying the interaction between different concentrations of Ca^{2+}, Sr^{2+} and Mg^{2+}. The general relation between transmitter release (in terms of m) and the concentration of a cation, A, combining with the 'Ca receptor', X, is described by

$$m = L\left(\frac{\beta_A[A]/K_A}{1+[A]/K_A}\right)^n,$$

where β_A is the relative potency of the A–X complex compared with that of the Ca–X complex, K_A is the dissociation constant of the $A + X \rightleftharpoons A\text{–}X$ reaction, n is the co-operative number (Dodge & Rahamimoff, 1967) and L is a constant which includes the concentration of X (see original derivation based on the law of mass action, Jenkinson, 1957). As shown by Meiri & Rahamimoff, $\beta_{Sr} = 0{\cdot}4$ gave a good fit to experimental observations at the neuromuscular junction, consistent with Sr^{2+} acting as a partial agonist of X, while very low β would apply for a competitive inhibitor, such as Mg^{2+} (Jenkinson, 1957). The interactions between Ca^2, Sr^{2+} and Mg^{2+} could thus be described by

$$m = L\left(\frac{[Ca]/K_{Ca} + 0{\cdot}4\,[Sr]/K_{Sr}}{1+[Ca]/K_{Ca}+[Sr]/K_{Sr}+[Mg]/K_{Mg}}\right)^n.$$

In the present experiments, this analysis has been applied to confirm the action of Sr^{2+} as a partial agonist at synapses in sympathetic ganglia. Values of K_{Ca}, K_{Mg} and K_{Sr} of 1·1 mM, 2·5 mM and 1·5 mM were assumed (from data obtained at the neuromuscular junction by Dodge & Rahamimoff, 1967; Meiri & Rahamimoff, 1971; Bennett, Florin & Hall, 1975). A value of $n = 0{\cdot}8$ during steady-state release at 10 Hz (unpublished personal observations) was derived from the slope of the relationship between log m and log [Ca] for three other ganglionic synapses. The experimental results shown in Fig. 5 indicate a reasonable fit to the prediction for the only synapse where sufficient data were collected. Recently published values for K_{Ca} and K_{Mg} of 0·8 mM and 0·8 mM derived for ganglionic synapses (Bennett, Florin & Pettigrew, 1976) give a slightly worse fit to these data.

The partial agonist action of Sr^{2+} (at concentrations of 2–5 mM) in potentiating release evoked in 0·5 mM-Ca^{2+} was confirmed at five other synapses (m increased 38·5 ± 8·0 %, S.E. of mean) (see Table 2 and Fig. 6*A*), while m was always depressed by the addition of Sr^{2+} to solutions

containing 2 mM-Ca^{2+} (m decreased $13{\cdot}3 \pm 2{\cdot}3$ %, S.E. of mean, $n = 3$; see Table 2 and Fig. 7*A*). The action of Sr^{2+} ions on 'phasic' transmitter release at ganglionic synapses was therefore indistinguishable from its reported action at the neuromuscular junction.

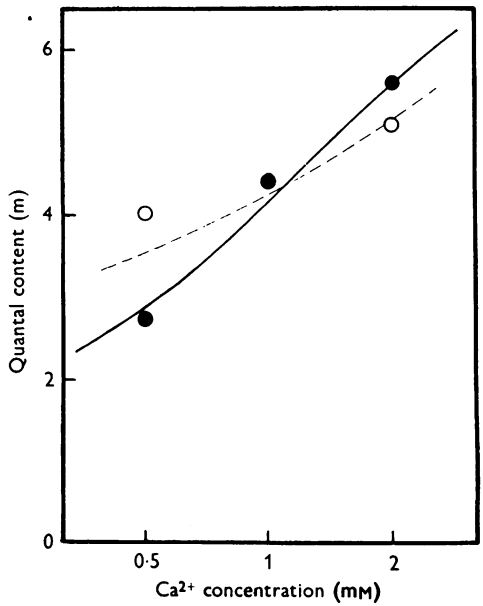

Fig. 5. Interaction between Sr^{2+} and Ca^{2+} ions. The mean values of quantal content, m, determined at a single synapse during steady-state release at 10 Hz in different Ca^{2+} concentrations (filled circles), and following the addition of 2·5 mM-Sr^{2+} (open circles). The continuous and dashed curves are the predicted relationships based on the equation given in the text (with $L = 9$). These results suggest that Sr^{2+} acts as a partial agonist of the receptor for Ca^{2+}.

Barium. In an attempt to distinguish between the possible actions of Ba^{2+} proposed in the previous section, the interaction between Ba^{2+} (2–5 mM) and either low or high concentrations of Ca^{2+} was studied. Changes in m were not significant ($-3{\cdot}0 \pm 9{\cdot}7$ %, S.E. of mean, $n = 3$) following the addition of Ba^{2+} to solutions containing 0·5 mM-Ca^{2+} (see Table 2 and Fig. 6*B*). The addition of Ba^{2+} in the presence of 2 mM-Ca^{2+} also had no effect on m ($-3{\cdot}2 \pm 2{\cdot}2$ %, S.E. of mean, $n = 5$) (see Table 2 and Fig. 7*B*). The simplest interpretation of these results is that Ba^{2+} does not compete with Ca^{2+} for X in the preganglionic nerve terminal, as it behaves neither as a partial agonist as does Sr^{2+}, nor as an antagonist as does Mg^{2+} (Bennett *et al.* 1976). The proposal (*a*) above therefore seems unlikely.

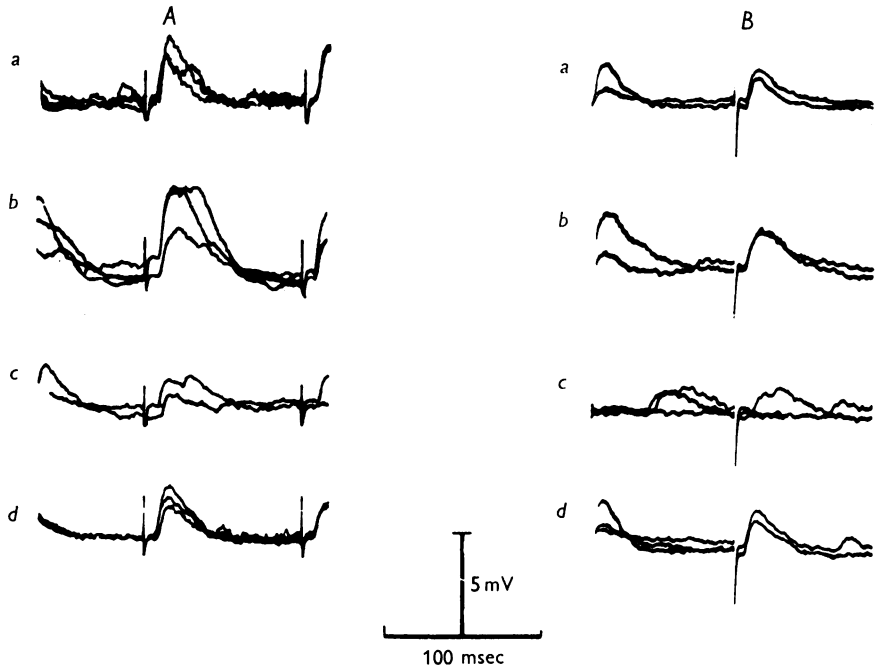

Fig. 6. The effects of Sr^{2+} (*A*) and Ba^{2+} (*B*) on ACh release in low concentrations of Ca^{2+}. Superimposed sweeps of e.p.s.p.s evoked during trains at 10 Hz. *A*,*B*, from different synapses. Bathing solutions contained in *A*: *a*, 0·5 mM-Ca^{2+}; *b*, 0·5 mM-Ca^{2+} + 5 mM-Sr^{2+}; *c*, 0-Ca^{2+}, 5 mM-Sr^{2+}; *d*, return to 0·5 mM-Ca^{2+}; in *B*: *a*, 0·5 mM-Ca^{2+}; *b*, 0·5 mM-Ca^{2+} + 5 mM-Ba^{2+}; *c*, 0-Ca^{2+}, 5 mM-Ba^{2+}; *d*, return to 0·5 mM-Ca^{2+}. While Sr^{2+} potentiated release in 0·5 mM-Ca^{2+}, Ba^{2+} had no detectable effect.

Effects on time course of e.p.s.p.s

Strontium. Prolongation by Sr^{2+} of spontaneous end-plate currents at the neuromuscular junction was attributed to a post-synaptic action on the response to ACh (Dodge *et al.* 1969). In the present experiments, prolonged e.p.s.p.s were observed following the addition of Sr^{2+} (see Fig. 6*A*). As an increase in mean min. e.p.s.p. amplitude usually occurred at the same time, this was initially attributed to the increase in R_{ln} (see above), and estimates of m were assumed to be unaffected. It seemed possible to test this assumption by analysing the time course of the e.p.s.p.s before and after addition of Sr^{2+}, using the method described by Edwards, Hirst & Silinsky (1976).

The time constant of decay of the e.p.s.p. in most ganglion cells was identical to the membrane time constant (see McLachlan, 1974); in the small proportion of cases this was not so, the synaptic current did not appear to follow the simple relationship used in the analysis of Edwards

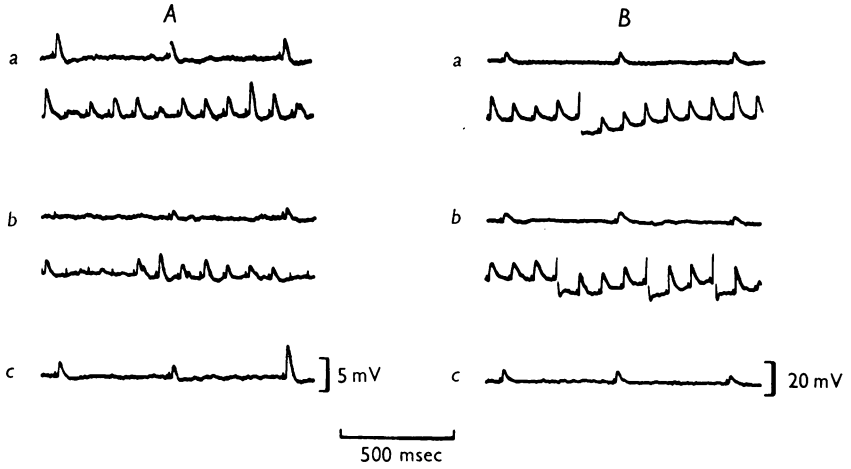

Fig. 7. The effects of Sr^{2+} (*A*) and Ba^{2+} (*B*) on ACh release in high concentrations of Ca^{2+}. Trains of e.p.s.p.s evoked during stimulation at 2 and 10 Hz. *A*, *B* from different synapses. Bathing solutions contained in *A*: *a*, 2 mM-Ca^{2+}; *b*, 2 mM-Ca^{2+} + 5 mM-Sr^{2+}; *c*, return to 2 mM-Ca^{2+}; in *B*: *a*, 2 mM-Ca^{2+}; *b*, 2 mM-Ca^{2+} + 5 mM-Ba^{2+}; *c*, return to 2 mM-Ca^{2+}. While Sr^{2+} depressed release in 2 mM-Ca^{2+}, Ba^{2+} had no detectable effect. Increased numbers of action potentials in the presence of Ba^{2+} resulted from a reduction in threshold (see Results).

et al. (1976) (personal observations; see also Martin & Pilar, 1963). Therefore, records of 'phasic' and spontaneous min. e.p.s.p.s were sampled from cells in which the two time constants were identical in solutions containing Ca^{2+}. From these records and the relevant values of R_{in} and C, it was possible to derive the time course of the synaptic current (see Fig. 8). This synaptic current was then used to predict the time course of the e.p.s.p.s evoked at the same synapse following the addition of Sr^{2+} and the consequent change in R_{in}. The shapes of both min. e.p.s.p.s and 'phasic' e.p.s.p.s recorded in the presence of Sr^{2+} were reasonably well predicted in this way (Fig. 8*A*), although there was a small prolongation of the falling phase of the 'phasic' response. From these data, it seems that, other than their effects on R_{in}, Sr^{2+} ions in the concentrations used in this study (2–5 mM) have only a small effect on other post-synaptic properties. It is possible that the local concentration of Sr^{2+} applied by iontophoresis in the experiments of Dodge *et al.* (1969) may have been somewhat higher.

A further result obtained using the analysis of Edwards *et al.* (1976) is an independent estimate of the expected change in the peak e.p.s.p. amplitude for a given synaptic current following a change in R_{in}. The results for three synapses following the addition of Sr^{2+} are compared with the observed changes in mean min. e.p.s.p. amplitudes in Table 3. It

TABLE 2. Effects of the addition of Sr^{2+} or Ba^{2+} on quantal content during steady-state release at 10 Hz in different Ca^{2+} concentrations

Concentration of		Quantal content (m)*	
Ca^{2+}	Sr^{2+}	Control	Test
0·5	2	6·21	7·03
0·5	5	3·23	5·38
0·5	5	3·40	4·93
0·5	5	2·1	2·75
0·5	5	7·82	9·70
0·5	2·5	2·72	4·11
0·5	5	2·52	5·19
2	5	4·62	3·84
2	5	6·69	5·78
2	2·5	5·59	5·10
Ca^{2+}	Ba^{2+}	Control	Test
0·5	2	1·76	1·37
0·5	2	2·61	2·69
0·5	2	3·85	4·24
0·5	5	3·91	4·32
2	5	4·06	3·86
2	5	7·1	7·14
2	5	4·64	4·32
2	5	12·83	13·21
2	5	5·86	5·39

* 'Control' values obtained in the appropriate [Ca^{2+}] before the addition of either Sr^{2+} or Ba^{2+} ('test' solution). Each set of values derived from a different cell except those indicated by brackets.

would be expected that the amplitude of both min. e.p.s.p.s and 'phasic' e.p.s.p.s would be changed by a similar proportion if Sr had no effect on either the time course or intensity of synaptic current resulting from the release of one quantum of transmitter, and if the amplitudes of 'phasic' e.p.s.p.s were small compared to the driving potential for the transmitter (see Methods). The reasonable agreement between the predictions suggests that significant errors in the estimate of m were not derived from the change in R_{in}.

Barium. E.p.s.p. time courses following the addition of Ba^{2+} were also analysed. The results (Fig. 8*B*) are rather different from those obtained in Sr^{2+}, and show that, in Ba^{2+}, while the min. e.p.s.p. shape compares well with the prediction, the 'phasic' e.p.s.p. is prolonged (see also Figs. 6*B* and 7*B*). It seems possible that this results from a prolongation of the preganglionic nerve impulse which might be expected to prolong the time course of transmitter release.

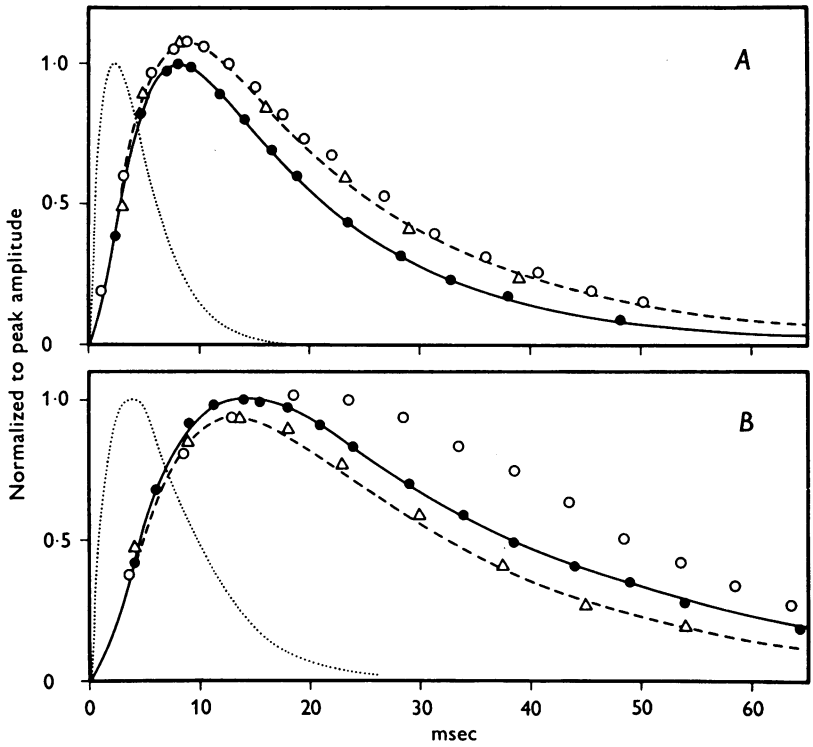

Fig. 8. Time course of e.p.s.p.s in the presence of Sr^{2+} and Ba^{2+}. Normalized time courses of synaptic current (dotted lines) and of e.p.s.p.s (continuous lines) are shown, together with the membrane potential changes (filled circles) recorded in 2 mM-Ca^{2+} from which these were calculated. *A*, data from one synapse where the addition of 5 mM-Sr^{2+} increased R_{in}: the predicted e.p.s.p time course (dashed line) is fitted by both the observed min. e.p.s.p. (open triangles) and the 'phasic' e.p.s.p. (open circles), normalized to the peak of the prediction. *B*, data from another synapse where the addition of 5 mM-Ba^{2+} decreased R_{in}: the predicted e.p.s.p. time course is well fitted by the observed min. e.p.s.p., but the peak of the 'phasic' e.p.s.p. is delayed and its shape is distorted. These results are consistent with the ideas that the major post-synaptic effects of Sr^{2+} and Ba^{2+} are on R_{in}, while the 'phasic' release of transmitter is prolonged by Ba^{2+}.

Predicted changes in the amplitude of the response to the same synaptic current are shown in Table 3; the mean min. e.p.s.p. amplitudes compared reasonably with these predictions. It should be noted that the error in the estimate of the mean min. e.p.s.p. amplitude is likely to be large in the presence of Ba^{2+}, frequencies of min. e.p.s.p.s being so high that distinction between unit and multiple responses was often difficult (see Bornstein, 1975).

TABLE 3. Comparison of peak amplitude changes predicted for a given synaptic current following a change in R_{in} and observed changes in mean min. e.p.s.p. amplitude

	Predicted (%)	Observed (%)
Sr	+7·8	+9·1
	+4·7	+3·5
	+25·8	+35·3
Ba	+11·5	+20·9
	−6·0	−8·9

Effects of Ca^{2+}, Sr^{2+} and Ba^{2+} on min. e.p.s.p. release during trains o impulses

Calcium. Repetitive stimulation of preganglionic axons increases the frequency of min. e.p.s.p.s (Blackman & Purves, 1969; McLachlan, 1975*a*). The results for one synapse in which this response was examined over a range of Ca^{2+} concentrations (Fig. 9) do not show a direct relationship between Ca^{2+} concentration and the frequency of min. e.p.s.p.s generated during repetitive stimulation, in contrast to previous reports at the neuromuscular junction (Hurlbut, Longenecker & Mauro, 1971; Rotshenker, Erulkar & Rahamimoff, 1976; see however Fig. 3 of Crawford, 1974). Furthermore, the averaged results in Table 4 also show that min. e.p.s.p. frequency during high-frequency stimulation was not higher in 2 mM-Ca^{2+} than in 0·5 mM-Ca^{2+}.

Strontium. Acceleration of min. e.p.s.p. frequency was more marked in the presence of Sr^{2+} (see, for example, Fig. 6*A*). The effect was greater in 0·5 mM-Ca^{2+} than in 2 mM-Ca^{2+} (Table 4), and was also dependent on the concentration of Sr^{2+} when this was examined in a single cell (Fig. 9) (see also Dodge *et al.* 1969). However, as it was usually possible to test only one concentration of Sr^{2+} at each synapse, the data for different Sr^{2+} concentrations have been pooled in Table 4; these results are from the same experiments listed in Table 2. This potentiation of 'residual' release occurs independently of the action of Sr^{2+} on 'phasic' release (cf. Fig. 5 and Table 2).

Barium. Ba^{2+} was almost twice as potent as Sr^{2+} in increasing the frequency of min. e.p.s.p.s evoked during trains of impulses, and again the effect was greater at the lower Ca^{2+} concentration (Table 4). These results have also been pooled for different concentrations of Ba^{2+} (as described above for results with Sr^{2+}). It should be noted that measurement of these high frequencies are extremely inaccurate; no attempt was made to allow for 'multiquantal' responses, so that the changes have probably been underestimated. These observations confirm the suggestion raised above

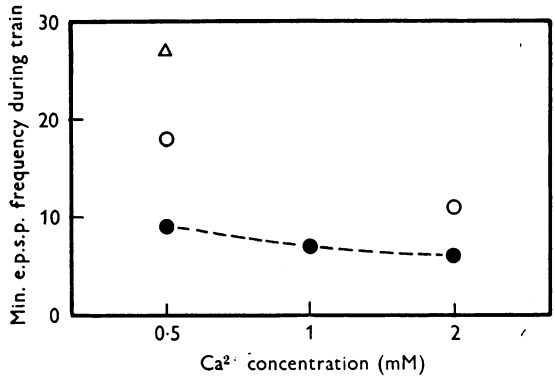

Fig. 9. The effect of Sr^{2+} on the frequency of min. e.p.s.p.s during tetanic stimulation. Average min. e.p.s.p. frequency during 100 impulses at 10 Hz in different Ca^{2+} concentrations (filled circles), and following the addition of 2·5 mM-Sr^{2+} (open circles) and 5 mM-Sr^{2+} (open triangle). Results from the same synapse as Fig. 5. The dashed line indicates an apparently inverse relationship between min. e.p.s.p. frequency and Ca^{2+} concentration under these conditions. Sr^{2+} always potentiated the 'residual' release of transmitter, the effect being greater at the higher Sr^{2+} concentration.

TABLE 4. The potentiation of 'residual' min. e.p.s.p.s by Sr^{2+} and Ba^{2+}

$[Ca^{2+}]$ (mM)	Frequency of stimulation (Hz)	f (Hz)	$f_{Sr}:f_{Ca}$	$f_{Ba}:f_{Ca}$
0·5	2	0·59 ± 0·20 (n = 7)	2·67 ± 0·74 (n = 4)	3·70 ± 0·88 (n = 3)
	10	5·05 ± 0·93 (n = 9)	2·32 ± 0·26 (n = 6)	4·32 ± 0·60 (n = 3)
2	2	0·79 ± 0·22 (n = 8)	1·43 ± 0·13 (n = 3)	2·67 ± 0·50 (n = 5)
	10	4·77 ± 0·90 (n = 8)	1·62 ± 0·23 (n = 3)	2·65 ± 0·42 (n = 5)

f is the frequency of min. e.p.s.p.s generated during trains of 100 impulses at the frequencies indicated. $f_{Sr}:f_{Ca}$ and $f_{Ba}:f_{Ca}$ represent the ratios of the frequency generated after the addition of either Sr^{2+} (2–5 mM) or Ba^{2+} (2 or 5 mM) to the frequency in the appropriate $[Ca^{2+}]$. Values are the means ± S.E. of mean.

that Ba^{2+} is a powerful stimulant of 'residual' release, while it is apparently without effect on the 'phasic' release receptor.

DISCUSSION

The 'Ca receptor' in preganglionic nerve terminals which initiates the 'phasic' release of transmitter is weakly activated by Sr^{2+} ions, in accord with previous reports for the neuromuscular junction (Meiri & Rahamimoff, 1971). Ba^{2+} ions, rather surprisingly, do not appear to compete with Ca^{2+} for this receptor. Substitution of Ca^{2+} with Ba^{2+} completely abolished 'phasic' e.p.s.p.s provided that a period of washing in Ca^{2+}-free solution preceded the introduction of Ba^{2+}. A partial agonist might have been expected to produce substantial inhibition of release at high Ca^{2+} concentrations, but this was never observed. It seems possible that direct transfer from Ca^{2+} solutions into Ba^{2+} solutions prevents the displacement of some of the Ca^{2+} from the tissue, thus maintaining some 'phasic' release; it is unclear whether retention of Ca^{2+} occurs at an intracellular or an extracellular location (see Baker, 1972; Hubbard, 1973).

Since Ba^{2+} caused depolarization of the ganglion cells, it might be expected to have depolarized the preganglionic nerve terminals. However, no evidence of such depolarization was obtained in the present experiments. It seems unlikely that there was a significant reduction in the amplitude of nerve terminal action potentials in view of the failure of Ba^{2+} to modify e.p.s.p.s evoked in solutions containing 0·5 or 2 mM-Ca^{2+}. In addition, both Ba^{2+} and Sr^{2+} (which did not depolarize the ganglion cells) produced increases in 'residual' release during trains of stimuli. Furthermore, no maintained changes in resting release of ACh were detected following the introduction of 2 or 5 mM-Ba^{2+} (see also Douglas *et al.* 1961); a small transient increase in min. e.p.s.p. frequency was observed, but this also occurred after the addition of Sr^{2+}. The failure to detect maintained changes in resting min. e.p.s.p. frequency following the addition of Ba^{2+} in the presence of Ca^{2+} is not in agreement with the reported potentiation of spontaneous release of miniature end-plate potentials (m.e.p.p.s.) at the neuromuscular junction by similar concentrations of Ba^{2+} in Ca^{2+} concentrations up to 5 mM (Laskowski & Thies, 1972). It appears that, if preganglionic terminals are depolarized by Ba^{2+}, the effect must be small and would seem unlikely to account for the actions of Ba^{2+} described in this report.

Both Sr^{2+} and Ba^{2+} act more powerfully than Ca^{2+} to augment the frequency of min. e.p.s.p.s generated during trains of impulses (see also Rahamimoff & Yaari, 1973; Alnaes *et al.* 1974). This activation of 'residual' transmitter release appears to be independent of the effects of these ions on 'phasic' release, and is common to a number of other cations, namely Mg^{2+} (Hurlbut *et al.* 1971), Co^{2+} and Ni^{2+} (Kita & van der Kloot, 1973), all of which are antagonists of 'phasic' release. Similar relative

potencies of these cations in accelerating quantal release of transmitter are observed in the presence of the ionophore, X-537A (Kita & van der Kloot, 1976), suggesting that they act inside the nerve terminal. If the activation of 'residual' release also follows the movement of cations into the nerve terminal, it is possible that they enter by a relatively non-selective pathway, separate from that associated with the Ca-influx responsible for 'phasic' release (see Hurlbut *et al.* 1971). As Ba^{2+} powerfully stimulates 'residual' release but has no effect on 'phasic' release, it is clear that two separate mechanisms exist.

Nerve stimulation either at higher frequencies or for more prolonged periods is required to produce accelerations of m.e.p.p. frequency at the neuromuscular junction (Miledi & Thies, 1971; Hurlbut *et al.* 1971; personal observations) comparable to those reported here for preganglionic nerve terminals. These latter are of considerably smaller size than the nerve terminals in skeletal muscle, suggesting that the increased quantal release may result from the accumulation of some substance inside the terminal when repetitive stimulation is prolonged beyond physiological limits. It is conceivable that entry of divalent cations might follow a rise in internal Na^{+} concentration under these conditions (see Baker, 1972; Blaustein, 1974). However, at this stage, it is not possible to distinguish whether the divalent cations mentioned above displace Ca^{2+} from intracellular binding sites, spontaneous release being accelerated by a rise in intracellular free Ca^{2+} concentration (Baker, 1972), or whether they directly activate release sites with different properties from those involved in 'phasic' release.

The accelerated release of min. e.p.s.p.s during trains of impulses in Ba^{2+} solutions seems to be the most likely source of the maintained ACh output from perfused ganglia (Douglas *et al.* 1961); that is, increased numbers of quanta released asynchronously during supramaximal stimulation of the preganglionic nerve might be expected to provide amounts of ACh approximately equal to those evoked synchronously with each impulse in Ca^{2+} solutions. This form of transmitter release has previously been suggested to account for the second 'pool' of ACh which can be depleted from preganglionic nerve terminals (McLachlan, 1975*c*). It is of interest that the replacement of Ca^{2+} by Ba^{2+} stimulates the secretion of catecholamines from the adrenal medulla (Douglas & Rubin, 1964 *a*, *b*), vasopressin and oxytocin from the neurohypophysis (Ishida, 1968) and insulin from pancreatic β cells (Hales & Milner, 1968). This effect of Ba^{2+} might be explained by the combination of its depolarizing action (Douglas, Kanno & Sampson, 1967) with an activation of release similar to the action on nerve terminals described above. Thus, in comparing different secretory systems, it may be appropriate to consider whether discrepancies

from results predicted by analogy with 'phasic' transmitter release might be accounted for if release can occur by two mechanisms with different sensitivities to divalent cations.

I am extremely grateful to Professor Mollie Holman, Dr G. D. S. Hirst and Dr E. M. Silinsky for helpful discussion and comments on the manuscript.

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